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Identification, localization and metal-catalyzed induction of specific metallothionein isoforms expressed by the adult human lens

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Identification, Localization and Metal Catalyzed Induction of Specific Metallothionein Isoforms expressed by the Adult Human Lens

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Thesis

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Abstract

Identification, Localization and Metal Catalyzed Induction of Specific Metallothionein Isoforms expressed by the Adult Human Lens

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Purpose: Metallothioneins (MTs) are a large family of proteins involved in multiple protective pathways, including binding of toxic metals, free radical scavenging, and oxidative stress. We have previously detected increased expression of the MT IIA gene in age-related human cataractous lenses relative to normal, suggesting a role for MTs in the maintenance of lens transparency. The MT family consists of many closely related isoforms grouped into four classes (I-IV). As a first step towards defining the function of MTs in the lens, we sought to establish the range and expression patterns of those MT isoforms expressed by the adult human lens. We also sought to identify those toxic heavy metals that induce MT IIA in cultured human lens epithelial cells (HLEs).

Methods: Normal human lenses were microdissected into epithelia and fiber cells. Primers specific for individual MT isoforms were designed. MT transcripts were monitored in whole lenses, epithelia and fibers by RT-PCR and confirmed to be authentic by sequencing. MT protein levels were evaluated by immunoblotting and by immunostaining. Northern analysis, RT-PCR and quantitative mimic RT-PCR were used to characterize MT IIA transcript levels in cultured human lens epithelial cells (SRA 01/04) treated with $CuCl_2$, $ZnCl_2$ and $CdCl_2$ at sub-lethal concentrations known to induce MT in other tissues.

Results: Transcripts encoding MT classes I and II but not III or IV were detected in adult human lenses. In addition to MT IIA, five other MT transcripts were identified including IE, IF, IG, IH and IL. MT IIA was detected almost exclusively in the lens epithelium while the class I isoforms were detected at high levels in both lens epithelia and fibers. MT protein was detected almost exclusively in the lens epithelium. $CdCl_2$ and $ZnCl_2$, but surprisingly not $CuCl_2$, induced expression of MT IIA in HLEs. Levels of induction were dependent on metal concentration. The highest levels of MT IIA were detected after 8 hrs of Cd^{+2} treatment and 3 hours of $ZnCl_2$ treatment. $CdCl_2$ treatment induced MT IIA 10-20-fold above basal levels and $ZnCl_2$ induced MT IIA 2-4-fold above basal levels.

Conclusions: The present report establishes the spectrum of MTs expressed by the adult human lens, defines their spatial expression patterns in lens epithelia and fibers and demonstrates that MT protein is abundantly present in the lens epithelium. This work also establishes that MT IIA is highly and rapidly induced by $CdCl_2$ and $ZnCl_2$ but surprisingly not by $CuCl_2$ in cultured HLE cells. These data suggest a role for different isoforms of MT in different portions of the lens and also suggests that regulation of MT transcription may play an important role in lens homeostasis and cataract.

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INTRODUCTION

The role of the eye lens is to focus light on the retina where visual information is processed and transmitted to the brain. To carry out this function the lens has evolved an exquisite degree of transparency and numerous protective systems. The lens is an interesting tissue because, unlike many tissues, it grows throughout the lifetime of an individual and presents a model for how cumulative environmental insults impact biological systems. Based on these properties, the lens has classically served as a model for diverse processes including stress biology, protein biochemistry, development and aging.

The adult lens consists of a single layer of epithelial cells that cover many layers of fiber cells. The whole organ is contained in a secreted capsule. The fiber cells closest to the epithelium are called primary fiber cells, the next layer of fiber cells are called secondary fiber cells and the inner most layers are called tertiary fiber cells. Developmentally, the human lens forms from the invagination of the neural placode to form the optic cup. This invagination of the human neural ectoderm eventually pinches off, creating a spherical lens vesicle that is 0.2mm in diameter whose wall is about 40μm thick.[1] The vesicle is separated from the surface ectoderm, but is in protoplasmic continuity with the cells of the optic cup.[1] Formation of the lens vesicle is rapidly followed by elongation of the cells of its posterior wall. These cells are destined to become primary lens fibers. Formation of the definitive primary fiber cells is signaled by a progressive nuclear pyknosis, which spreads from the center of the fiber mass while the remaining epithelial cells revert back to a more cuboidal shape.[1] With the addition of secondary fiber cells resulting from division of cells from the equator, the primary fiber

mass becomes separated from the epithelium and occupies the center of the lens, known as the nucleus. At the end of development, there are two main cell types in the lens: the lens epithelium and the lens fibers (shown in Fig. 1).

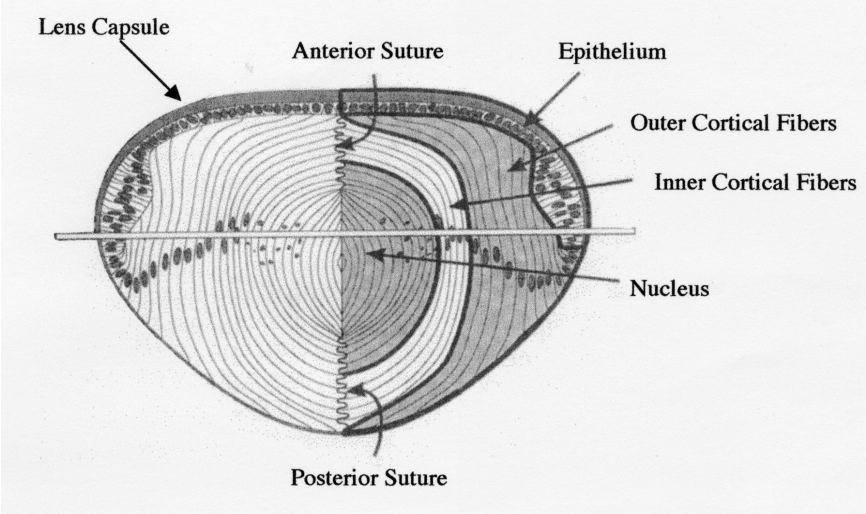

Fig. 1. Chicken embryo lens that shows different portions of the lens. This diagram was adapted from Beebe D, Vasiliev O, Guo J, Shui YB, and Bassnett S. Changes in adhesion complexes define stages in the differentiation of lens fiber cells. *IOVS* March 2001, Vol. 42, No. 3: 727-734.

The lens is covered by an unusually thick basal lamina called the lens capsule. This capsule is secreted by the lens epithelium during development. After formation of the primary fiber cells, only anterior epithelium persists, so the future thickening of the capsule is dependent on these epithelial cells.[2] Thus, the lens capsule is thickest in the anterior portion of the lens. The capsule is freely permeable to water, ions and other small molecules but offers a barrier to proteins greater in size than albumin (74kDa) and hemoglobin (67 kDa).[3]

The lens epithelium is made of cuboidal cells that form a monolayer covering the anterior subcapsular region to the lens equator. There are about 500,000 cells in the lens epithelium of a mature lens.[4] These cells possess large indented nuclei with two nucleoli

and a moderate number of organelles and these cells are responsible for the transcriptional activity of the lens.[1] The epithelial cells are connected to the fiber cells through infrequent gap junctions, and there is no significant barrier to extracellular flow between lens epithelial cells.[5,6] These gap junctions allow for the lens epithelium to respond to changes in the fiber cells through altering gene expression. Few mitoses occur in the central region of the lens epithelium with most mitotic events occurring in the germinative zone, which is pre-equatorial. This area of the epithelium is composed of highly ordered meridional rows of cells that can differentiate posteriorly into secondary lens fibers. Epithelial mitosis is associated with DNA synthesis, while fiber elongation is associated with increased transcritptional activity and RNA synthesis as specific membrane and structural proteins are needed.[1]

Fiber cells undergo a process of terminal differentiation in which the nuclei become pyknotic and finally disappear and cell organelles are lost. As the fiber cells differentiate, the chromatin clumps, the nuclear envelope disintegrates and the nuclear matter eventually disappears along with the organelles. Superficial lens fibers contain sparse organelles that are eventually lost from the deep cortex and lens nucleus. Lens fibers are ribbon-shaped cells that are hexagonal in cross-section. They have acutely angled short faces that connect the parallel broad faces and form the lateral membranes of the cells.[1] These fibers are joined together by ball and socket joints at their lateral angles and to some extent over their broad faces.[1] Fiber cells also have tongue and groove joints, which can be seen as surface corrugations at the narrow lateral faces of the fibers.[7] The nucleus fiber cells have been shown to posses elongated microvilli,[8,9] allowing the lens to flex to accommodate new fiber cells since they cannot slide over one another.

A major stress resistance mechanism involving the lens is oxidative stress protection. Many stresses, such as UV light exposure, result in reactive oxygen species being produced in the lens. A major player involved in free radical and other reactive oxygen species scavenging is glutathione. Glutathione is present in high concentration in the lens cytoplasm and about 12% of ATP produced in the lens is used to form glutathione protein. Glutathione levels decrease with age and levels are shown to fall in a disease of the lens known as cataract.[10] Another important protective pathway in the lens is the role of chaperone proteins. An example of a chaperone protein is alpha-crystallin, which is known to contribute to the refractive index and transparency in the lens. Alpha crystallin has been shown to prevent denaturation and facilitate the folding of other proteins. Another major mechanism is metal regulation, which is important in maintaining lens transparency. Examples of proteins involved in metal detoxification in the lens are ferritin and metallothionein. If a metal, such as copper, is not removed from the lens and forms deposits, it will disrupt the normal function of the lens and affect its transparency and refractive index. An example of this is the sunflower cataract, which is formed due to copper deposits[1].

Any opacity in the lens that causes light scattering is referred to as a cataract. Cataracts are the leading cause of world blindness and are the biggest cost for Medicare annually. Cataract can occur at any age but their incidence is associated with increasing age.[1] The average age of cataract onset in the United States population is 60-65. There are many factors that contribute to the development of cataracts and some of these factors include toxic heavy metals, oxidative stress, genetic factors, ions and osmotic disruptions. The first line of defense against these environmental insults is the lens epithelium and

since the lens epithelium constitutes 90% of the lens transcriptional activity, it is important for this tissue to protect the lens from insults by regulating gene expression involved in protective pathways.[1] Typically, when a cataract does form it is due to an aggregation of the protein in the lens fibers, which then can no longer maintain transparent function leading to light scattering. Since there are many causes for cataracts, the factors can be divided into two main categories: 1) ones that are due to genetic diseases; and 2) ones associated with environmental influences and aging (age-related cataract). This thesis focuses on factors that are involved with age related cataracts.

Our laboratory is interested in identifying those genetic and biochemical pathways involved in the protection against and development of age-related cataracts. Previously, we have found that Metallothionein IIA (MT IIA) is about 10 times over-expressed in cataractous human lens when compared to normal human lenses.[11] Metallothionein is a large family of small molecular weight (6-7 kDa), cysteine rich metal-binding proteins.[13] There are at least 16 different isoforms of MT that are grouped into four different classes designated I-IV and they are located on human chromosome 16.[13] MTs are approximately 60 amino acids long and contain 20 cysteine residues that coordinate up to 7 metal atoms.[14] MTs provide a reservoir for supplying zinc and copper in the biosynthesis of metalloenzymes and metalloproteins and are therefore produced at basal levels to support this homeostatic activity.[12] Thus, MTs may serve as a zinc-dispensing and collecting system that both protects the cellular constituents against fluctuations in zinc supply and modulates the action of zinc-dependent processes fundamental to cell activation in proliferation and differentiation.[12]

Metallothioneins are induced upon exposure to heavy metals and participate in heavy metal regulation and detoxification.[12,15] They are known to bind Zn, Cu, Co, Cd, Hg, Pb, Ag, Bi, Sn, Ni and Au.[12] Recent studies suggest that detoxification and regulation of heavy metals is not the only or even the primary function of MTs.[16] In addition to their metal binding properties, they are also free radical scavengers[17] whose synthesis is activated by steroids,[18,19] carcinogens,[20] chemicals that induce oxidative stress,[21] X-irradiation and UV-induced DNA damage.[22]

Metallothioneins are known to be involved in metal detoxification and scavenging of reactive oxygen species. They are known to be transcriptionally activated by UV light, reactive oxygen species, steroids and toxic metals.[12] In my work, there were two main goals: I) to identify those metallothionein isoforms that are expressed by the normal human lens; and II) to determine the induction levels of MT IIA induced by toxic heavy metals in cultured human lens epithelium.

Part I: Identification and Spatial Analysis of Metallothioneins Expressed by the Adult Human Lens

ABSTRACT

Purpose: Metallothioneins (MTs) are a large family of proteins involved in multiple protective pathways, including binding of toxic metals, free radical scavenging, and oxidative stress. We have previously detected increased expression of the MT IIA gene in age-related human cataractous relative to normal lenses, suggesting a role for MTs in the maintenance of lens transparency. The MT family consists of many closely related isoforms grouped into four classes (I-IV). As a first step towards defining the function of MTs in the lens, we sought to establish the range and expression patterns of those MT isoforms expressed by the adult human lens.

Methods: Normal human lenses were microdissected into epithelia and fiber cells. Primers specific for individual MT isoforms were designed. MT transcripts were monitored in whole lenses, epithelia and fibers by RT-PCR and confirmed to be authentic by sequencing. MT protein levels were evaluated by immunoblotting and by immunostaining.

Results: Transcripts encoding MT classes I and II but not III or IV were detected in adult human lenses. In addition to MT IIA, five other MT transcripts were identified including IE, IF, IG, IH and IL. MT IIA was detected almost exclusively in the lens epithelium while the class I isoforms were detected at high levels in both lens epithelia and fibers. MT protein was detected almost exclusively in the lens epithelium.

Conclusions: The present report establishes the spectrum of MTs expressed by the adult human lens, defines their spatial expression patterns in lens epithelia and fibers and demonstrates that MT protein is abundantly present in the lens epithelium.

INTRODUCTION

The vertebrate eye lens contains multiple protective and regulatory pathways to maintain its transparent function. Insult to these pathways is believed to result in cataract formation. Insulting agents include toxic metals, free radical formation and oxidative stress.[1, 22-27] We are interested in identifying those components of the human lens that protect against these insults.

One component, likely to play a major role in lens protection is the metallothioneins (MTs), which are a large family of closely related isoforms that have been implicated in numerous detoxification and protective pathways.[12] We have previously detected increased levels of MT IIA transcripts in pooled age-related human cataractous relative to normal lenses by RT-PCR differential display,[11] indicating a potential role for at least one member of the MT family in the maintenance of lens transparency.

MTs are 6-7kD metal binding proteins that are induced upon exposure to heavy metals and participate in heavy metal regulation and detoxification.[12] They are known to bind Zn, Cu, Co, Cd, Hg, Pb, Ag, Bi, Sn, Ni and Au.[12] Recent studies suggest that detoxification and regulation of heavy metals is not the only or even the primary function of MTs.[13] In addition to their metal binding properties, they are also free radical scavengers[17] whose synthesis is activated by steroids,[18,19] carcinogens,[20] chemicals that induce oxidative stress,[21] X-irradiation and UV-induced DNA damage.[22] They have been

reported to function as regulators of copper homeostasis,[28-30] as zinc donors for the zinc-dependent transcription factors,[31,32] as mediators of redox balance[33] and as protectors against reactive oxygen intermediates.[34-36]

The MT family is composed of multiple, separately-encoded isoforms[37] that are grouped into four physically distinct classes (called I- IV).[13] MTs I and II are the most widely expressed and are regulated coordinately in all tissues.[38,39] MT III is brain specific[14] and MT IV is expressed mainly in squamous epithelium.[40] In humans, single genes located on chromosome 16 and designated IIA, III and IV encode MT isoforms II-IV, while there are 13 separately-encoded MT I isoforms.[40]

Given the large number of MTs, and their different expression patterns, we have explored the range and spatial expression patterns of specific MTs expressed by the adult human lens as a first step towards elucidating their potential functions in the lens. We demonstrate that at least six different MT transcripts are expressed by the lens and these exhibit different patterns of expression in the lens epithelium compared to the lens fibers. We also demonstrate that MT protein is primarily present in the lens epithelium.

METHODS

Microdissection of human lenses. Normal human lenses from donors with no known history of metal exposure were obtained within 24-60 hours post-mortem and inspected under a dissecting scope to be free of opacity. The lens epithelium was surgically dissected away from the rest of the lens and contaminating fiber cells were removed as previously described.[11] Carefully washed surgical grade instruments and metal free solutions were used in all dissection and preservation procedures. The average age of lenses used in this study was 57 years (range 38-84) and the donors were 69% male.

Where indicated, four to eight lenses or lens sub-components were pooled for each experiment. This research was conducted under IRB exemption 4.

Design of oligonucleotides for RT-PCR. Oligonucleotides specific for individual MT isoforms were designed using the blast program and GeneBank data (National Library of Medicine, Bethesda, MD). The GeneBank accession numbers are MT IA (K01383), MT IB (M13484), MT IE (M10942), MT IF (M10943), MT IG (J03910), MT IH (X64834), MT IL (AJ011772), MT IIA (X00504), MT III (M93311) and MT IV (U07807). The sequences were MT IA (5'- GCCTCTCAACTTCTTGCTTG and 5'- GAC ATCAGGCACAGCAGCTG), MT IB (5'- GCCCTGACTTCTCATATCTTG and 5'- GG CACTTCTCTGATGAGCCTT), MT IE (5'- GCTCCAGCATCCCCTTTGCT and 5'- CA CATCAGGCACAGCAGCTG), MT IF (5'- GCTTCTCTCTTGGAAAGTCC and 5'- GG CATCAGTCGCAGCAGCTG), MT IG (5'- GCCTCTTCCCTTCTCGCTTG and 5'- GACATCAGGCGCAGCAGCTG), MT IH (5'- GAACTCCAGTCTCACCTCGG and 5'- GACATCAGGCACAGCAGCTG), MT IL (5'- GACTGCCTCTTCGCCTCTCC and 5'- CACATCAGGCACAGCAGCTG), MT IIA (5'-AAGTCCCAGCGAACCCG CGT and 5'- CAGCAGCTGCACTTGTCCGACGC), MT III (5'- GTTGCTTGGAG AAGCCCGTT CA and 5'- GCATAGGTGGCACTGAGCCA) and MT IV (5'- GCC GTGACAGCA CTGGAGCCT and 5'- GATTCATGCACTGTACAGACAC). The expected product sizes were: MT IA - 237 bp, MT IB - 229 bp, MT IE - 211 bp, MT IF - 226 bp, MT IG - 234 bp, MT IH - 213 bp, MT IL - 226 bp, MT IIA - 236 bp, MT III - 318 bp and MT IV - 294 bp. Control oligonucleotides were also designed to examine GAPDH, MIP26 and SPARC. The GeneBank accession numbers are GAPDH (NM_002046), MIP26 (U36308) and SPARC (J03040). The sequences were GAPDH

(5'- TCCACCACCCTGTTGCTGTA and 5'- TGTTCCAGTATGATTCCACCC), SPARC (5'- CCTGAGGCTGTAACTGAGAG AAAG and 5'- GTGGGAGG GGAAA CAAGAAGATAA) and MIP26 (5'- GCTTGGCCCTGGCTACACTGGT and 5'- TGA GCCGGGGGAAGAGAAGAA). The expected product sizes were: GAPDH - 840 bp. SPARC - 419 bp and MIP26 - 538 bp. Oligonucleotides were designed to cross intron-exon boundaries to distinguish between DNA and RNA.

Reverse Transcription-Polymerase Chain Reaction. Total RNA was prepared from pooled whole lenses, lens epithelium and lens fibers by phenol-guanidinium isothiocyanate extraction[11]. RT-PCR was performed with indicated amounts of RNA using the one-step system according to the manufacturer (GIBCO-BRL, Gaithersburg, MD). As control, reactions were also performed in the presence and absence of reverse transcriptase using amplitaq polymerase (Perkin-Elmer Cetus, Wellesley, MA). Products were separated by gel electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining.

RT-PCR using universal recognition primers. RT-PCR was also performed using primers designed to recognize all MT I and MT II isoforms. The conditions for this procedure were identical with the above procedure. The sequences of the MT universal primers are (5'- ATGGACCCCAACTGCTCCTG and 5'- GCAGCAGCTCTTCTTGC AGG). The expected product size for these primers is 102bp.

Cloning of PCR products. Specific RT-PCR products were excised and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA), and cloned into Topo TA vectors as specified by the manufacturer (Invitrogen, Carsbad, CA).

Identification of specific transcripts. Transcripts were sequenced using automated fluorescent cycle DNA sequencing on an Applied Biosystems 373a DNA sequencer using Applied Biosystems sequencing software (Perkin Elmer, Applied Biosystems, Wellesley, MA). Sequences were analyzed using the BLAST algorhythm with GeneBank data (National Library of Medicine). Subsequent alignments were performed using the MegAlign program contained in Lasergene software (DNASTAR, Inc., Madison, WI).

Western Blot Analysis. Indicated amounts of protein were denatured by boiling in 10% SDS buffer (10% w/v SDS; 0.5 M Tris-HCL, pH 6.8, 5% [v/v] 2-mercaptoethanol, 5% [v/v] glycerol), resolved by electrophoresis on 12% SDS-polyacrylamide gels and transferred (60V for a half hour in 12 mM Tris-HCL, 96 mM glycine, 15% methanol) to nitrocellulose filters. The resulting blots were blocked overnight in Superblock (Pierce, Rockford, IL) according to the manufacturer. The blot was washed three times with PBS over 15 min., incubated with a 1:200 dilution of mouse anti-metallothionein antibody (StressGen, Victoria, British Columbia, Canada) and washed three times in PBS containing 0.1% Tween 20. Immunoreactive metallothionein was visualized using ECL western blotting reagents (Amersham-Pharmacia, Piscataway, NJ) as specified by the manufacturer. Identical procedures were carried out with purified rabbit liver MT (Sigma, St. Louis, MO) as control.

Immunostaining. An 18 year-old female human lens (less than 24 hours post-mortem) was fixed in 4% paraformaldehyde in PBS overnight, followed by cryoprotection overnight in 30% sucrose in PBS prior to embedding. Frozen sections (14 μm) were prepared and air-dried. Sections were blocked for one hour at room temperature in DMEM, 10% fetal calf serum, 1% goat serum, and 0.1% Triton X-100. Sections were

incubated overnight with a 1:400 dilution of anti-metallothionein antibody (DAKO, Carpinteria, CA) in blocking solution at 4°C. After five washes with 0.1% Tween 20 in PBS, sections were incubated with streptavidin-conjugated secondary antibody (Vector Lab, Burlingame, CA) and were visualized using the Vectastain Elite Kit as specified by the manufacturer. Sections were counter-stained with hematoxylin. Identical procedures were carried out in the absence of primary antibody as control.

RESULTS

Identification of specific metallothionein transcripts.

Expression of 11 different MT isoforms was examined by RT-PCR analysis of whole lens RNA. The data are shown in Fig.2A. High levels of transcripts specific for MT isoforms IE[41] (211bp, lanes 5 and 6), IF[41] (226bp, lanes 7 and 8), IG[42] (234bp +270bp upper band, lanes 9 and 10), IH[43] (213bp, lanes 11 and 12) and IIA[44] (236bp, lanes 17 and 18) were detected by this analysis. By contrast, lower amounts of product were detected using primers designed to recognize isoforms IA[45] (237bp expected + 260bp upper band, lanes 1 and 2) and IL[46] (226bp + 250bp upper band, lanes 15 and 16). No product was detected for isoforms IB[47] (lanes 3 and 4), III[14] (lanes 19 and 20) or IV[40] (lanes 21 and 22). As control, high levels of GAPDH were detected in control reactions (840bp, lanes 13 and 14). Single products were detected in all reactions except for IA, IL and IG, which also contained a (270bp) upper band of unexpected size. All reactions yielding high levels of product were specific for the presence of reverse transcriptase (Fig. 2B, lanes 1-14).

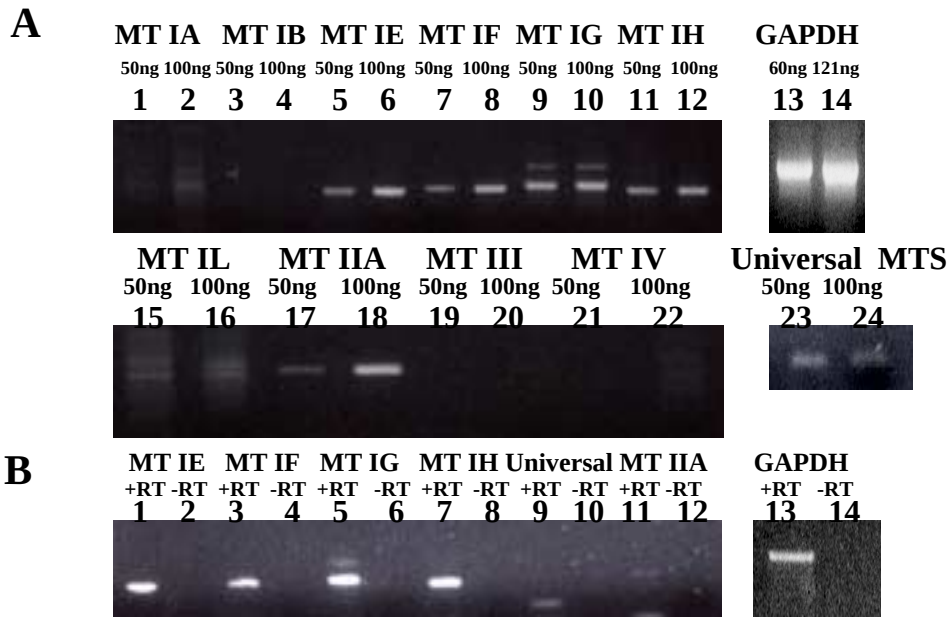

Fig. 2: Expression of metallothionein isoforms in the adult human lens. **A**. Ethidium bromide stained gel showing indicated MT transcripts (lanes 1-12 and 15-22) amplified by RT-PCR (32 cycles) using isoform-specific primers and indicated amounts of whole lens RNA. Transcript sizes are: MT IA (237bp), MT IE (211bp), MT IF (226bp), MT IG (234bp) MT IH (213bp), MT IL (226bp) and MT IIA (236bp). Also shown are RT-PCR products obtained with primers (Universal MTs) designed to recognize all class I and II MT isoforms (102bp; lanes 23 and 24) and, as control, GAPDH (840bp; lanes 13 and 14). **B**. Ethidium bromide stained gel showing indicated transcripts (lanes 1-14) amplified in the presence (+RT) or absence (-RT) of reverse-transcriptase using 50ng of whole lens RNA and 32 PCR cycles.

To confirm the identities of the resulting transcripts, products of the expected size that were specific for isoforms IA, IE, IF, IG, IH, IL and IIA were cloned and sequenced. Analysis of 3 separately isolated clones confirmed these transcripts to be authentic except for IA, which contained a mixture of transcripts encoding IG and IH. The identities of the larger bands detected in the IA, IG and IL reactions were not determined.

To further characterize the specific MT isoforms expressed by the lens, RT-PCR was performed using primers (universal primers) designed to recognize and amplify all class I and class II MT transcripts. The forward primer is specific for exon 1 and the reverse primer overlaps exons 2 and 3 of the MT class I and II coding sequences. The forward primer sequence is identical to the corresponding sequences of isoforms IA, IF, IG, IH, and IL and differs by only one base pair with the corresponding sequences of

isoforms IB, IE, IR and IIA. The reverse primer sequences is identical to the corresponding sequences of isoforms IA, IF, IG, IH, IL and IR and differs by only one base pair with the corresponding sequences of IB, IE and IIA.

The universal primers were designed to produce a 102bp RT-PCR product. Purification and cloning of this product, followed by sequencing and identification of individual clones, should identify the spectrum of class I and II transcripts present in the lens. The number of individual clones divided by the total number of clones should approximate the relative frequency of each transcript expressed by the lens.

RT-PCR of pooled whole lens RNA using the universal primers yielded a single band of the expected size (Fig. 2A (102bp); lanes 23 and 24) that was dependent on the presence of reverse-transcriptase (Fig. 2B; lanes 9 and 10). The 102bp band stained less intensely than the isoform-specific bands (compare Fig. 2, lanes 23 and 24 to lanes 5-12, 17 and 18). This most likely is the result of reduced amplification efficiency of the 102bp product since the universal primers are likely to have reduced binding efficiency relative to the isoform-specific primers. The 102bp product was excised from the gel, purified and cloned. Seventy-five separately isolated clones were identified by sequencing (Table 1).

MT Isoforms Detected	No. of Clones Identified	Proportion of Total Clones
MT IE	**1**	**1%**
MT IF	**5**	**7%**
MT IG	**8**	**11%**
MT IH	**3**	**4%**
MT IL	**51**	**68%**
MT IIA	**7**	**9%**

Table 1: Identities and Relative Proportions of MT Transcripts Amplified from whole Lens RNA with Universal Recognition RT-PCR Primers.

Consistent with the identities of transcripts detected using the isoform-specific primers (Fig. 2), six MT isoforms were identified by this analysis including MTs IE, IF, IG, IH, IL and IIA.

Analysis of metallothionein transcripts in microdissected lens epithelia and fibers.

To examine the spatial expression patterns of the identified transcripts, the levels of five MT transcripts (IE, IF, IG, IH and IIA) were compared by RT-PCR analysis using pooled RNA obtained from microdissected lens epithelium and lens fibers. To control for cross-contamination between the lens epithelia and fiber RNA preparations, the epithelium-specific transcript SPARC[48] and the fiber-specific transcript MIP 26[49] were also examined.

Transcripts encoding MTs IE, IF, IG and IH (lanes 1-8) were detected in both lens epithelium and lens fibers (Fig. 3A). By contrast, MT IIA transcripts (lanes 9 and 10) were detected only in the lens epithelium (Fig. 3A). Using higher amounts of RNA and increased numbers of PCR cycles, low amounts of MT IIA were detected in the fibers (data not shown). As found with whole lens RNA (Fig. 2, lanes 9 and 10), a second higher molecular weight band than the authentic MT IG band was also observed (Fig. 3, lane 5). Although the identity of this band was not determined, it is interesting to note that it is confined to lens epithelial RNA preparation (compare Fig. 3, lanes 5 and 6).

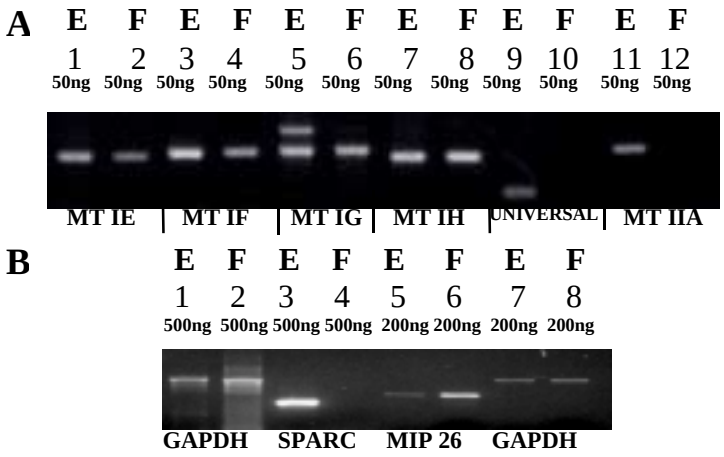

Fig. 3: Expression of metallothionein isoforms in pooled lens epithelia and lens fibers. A. Ethidium bromide stained gel showing indicated MT transcripts (lanes 1-10) amplified by RT-PCR (32 cycles) using isoform-specific primers and 50ng of RNA isolated from lens epithelia (E) or fibers (F). B. Ethidium bromide stained gel showing indicated control transcripts (lanes 1-4) amplified by RT-PCR (32 cycles) using SPARC- or MIP 26-specific primers and indicated amounts of (E) epithelial or fiber (F) RNA.

As expected, the SPARC control was detected only in the lens epithelial RNA preparation (Fig. 3B, lanes 1 and 2). Correspondingly, the MIP 26 control was almost entirely restricted to the lens fiber RNA preparation (Fig. 3B, lanes 3 and 4), although trace amounts of MIP 26 were detected in the epithelial preparation indicating possible contamination of lens epithelial RNA with fiber RNA. Since greater levels of almost all transcripts were found in the lens epithelium relative to the fibers, and no fiber-specific gene expression was detected, it is unlikely that trace contamination of epithelial RNA with fiber RNA would significantly alter the present results.

Analysis of metallothionein transcripts in individual lenses.

It is possible that MT expression might vary between individual lenses. Since the previous experiments (Figs. 2 and 3) examined MT levels in pooled lenses, the expression of two MT isoforms (MT IG and MT IIA) was examined using equal amounts of RNA isolated from individual lens epithelia or fibers (Fig. 4). The levels of both MT

isoforms were variable in individual lens epithelia and fibers. This variability does not appear to be related to age or sex. Interestingly, the levels of MT IIA

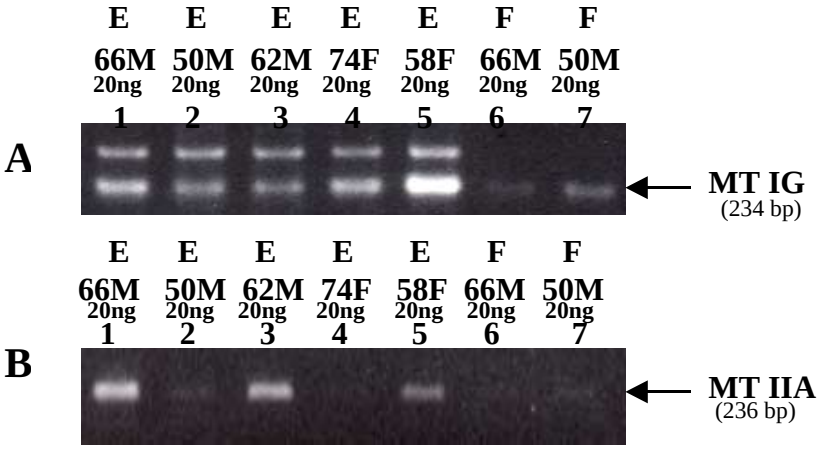

Fig. 4: Expression of MT IG and MT IIA in individual lens epithelia and fibers. A. Ethidium bromide stained gel showing MT IG transcripts amplified by RT-PCR (25 cycles) using 20ng of epithelia (E) or fiber (F) RNA. Donor ages and sexes (M, male and F, female) are indicated. B. Ethidium bromide stained gel showing MT IIA transcripts amplified by RT-PCR (25 cycles) using 20ng of epithelia (E) or fiber (F) RNA. Donor ages and sexes (M, male and F, female) are indicated.

and MT IG were not proportional in the same lens epithelia (compare Fig. 4A to Fig. 4B, lanes 2, 4 and 5). The levels of MT IG were lower in lens fibers than in epithelia (Fig. 4A, lanes 6 and 7) and trace amounts of MT IIA were detected in lens fibers (Fig. 4B, lanes 6 and 7). MT IIA exhibited greater variation between individual lenses than did IG (compare Fig. 4, A and B) indicating different levels of variation between specific MT isoforms in individual lenses.

Spatial analysis of metallothionein protein.

To evaluate the corresponding levels of MT protein in the lens, western analysis was performed using a MT-specific antibody and equal amounts of extracts prepared from pooled whole lens, lens epithelia and lens fibers. Since antibodies specific for individual MT isoforms are not available as a result of the very high degree of amino acid

sequence identity between MT classes I and II (at most a one amino-acid difference), an antibody recognizing both MT classes[50] was chosen for this analysis.

Consistent with the reported molecular weight for MT, a 6-8kD immunoreactive band co-migrating with the corresponding 6-8kD purified MT band (lane 4) was detected with epithelial (lane 3) and whole lens extracts (lane 1)(Fig. 5A). A second 16-20kD band is also visible in the purified MT (lane 4), lens epithelia (lane 3) and weakly in whole lens (lane 1)(Fig. 5A). Although the identity of this band is not known, it is likely that it contains cross-linked MTs[50] that may also represent the higher immunoreactive bands (35-50kD) restricted to the purified MT control (lane 4). In contrast to the whole lens or lens epithelial protein extracts, no MT was detected using equal amounts of lens fiber extracts (lane 2) indicating that MT protein is confined to the lens epithelium.

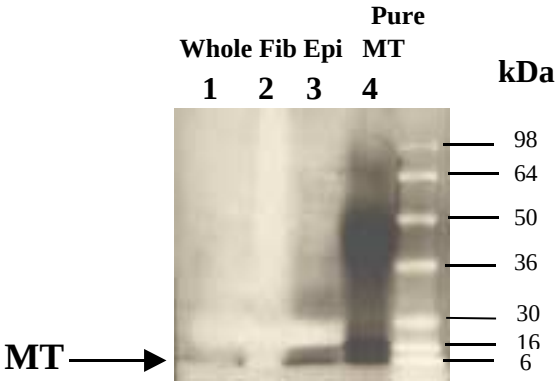

Fig. 5: Immunoblotting of lens extracts with metallothionein-specific antibody. Shown is the autoradiogram of the corresponding blot. Lane 1 contains 1.25ug of whole, lane 2, 1.25ug of fiber (Fib) and lane 3, 1.25 ug of epithelial (Epi) lens extracts. Lane 4 contains 50 ng of purified rabbit liver MT protein. Indicated is the 6-8kD immunoreactive MT band and the positions of molecular weight standards.

To further explore the levels of MT protein in specific lens sub-regions, immunostaining was performed with a second antibody recognizing MT class I and II isoforms (Fig. 6A).[51] Represented is a portion of the lens between the anterior and

peripheral regions. Consistent with the immunoblotting results (Fig. 5), MT protein was detected at significantly higher levels in the lens epithelium than in the lens fibers. No staining was detected in the lens capsule (Fig. 6A). It is interesting to note that the weak staining in the lens fibers appears to be restricted to undifferentiated fibers. As control, no staining was detected in the absence of primary antibody (Fig. 6B).

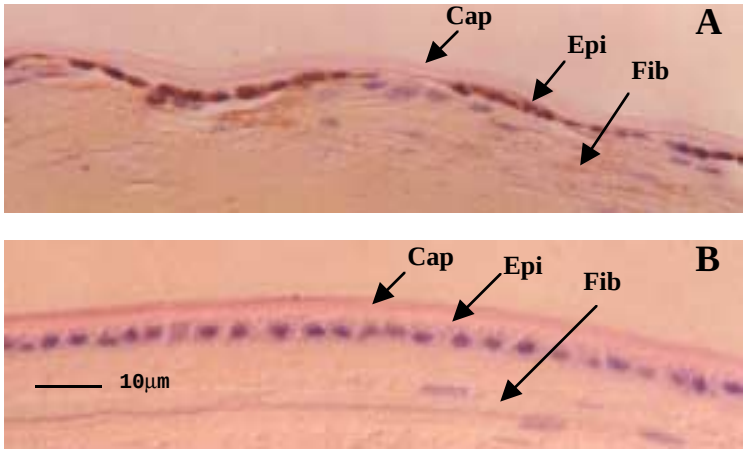

Fig. 6: Immunostaining of adult human lens with metallothionein-specific antibody. An 18 year-old female human lens was immunostained with (**A**) anti-metallothionein antibody or **(B**) secondary antibody alone. Indicated are the lens capsule (Cap), lens epithelium (Epi) and lens fibers (Fib). The 10 μm bar denotes 10 microns.

Part II: Induction of MT IIA by Toxic Heavy Metals in Cultured Human Lens Epithelial Cells

ABSTRACT

Purpose. MT's are major detoxification proteins that are induced by toxic metals in many non-ocular tissues. We have previously detected cataract and epithelium-specific expression of human MT IIA. The purpose of the present study was to identify those heavy metals that induce MT IIA in cultured human lens epithelial cells.

Methods. Northern analysis, RT-PCR and quantitative mimic RT-PCR were used to characterize MT IIA transcript levels in cultured human lens epithelial cells (SRA 01/04) treated with $CuCl_2$, $ZnCl_2$ and $CdCl_2$ at sub-lethal concentrations known to induce MT in other tissues.

Results. $CdCl_2$ and $ZnCl_2$, but not $CuCl_2$, induced expression of MTIIA in HLEs. Levels of induction were dependent on metal concentration. The highest levels of MT IIA were detected after 8 hrs of $CdCl_2$ treatment and 3 hours of $ZnCl_2$ treatment. $CdCl_2$ treatment induced MT IIA 10-20-fold above basal levels and $ZnCl_2$ induced MT IIA 2-4-fold above basal levels.

Conclusions. MT IIA is highly and rapidly induced by $CdCl_2$ and $ZnCl_2$ but surprisingly not by $CuCl_2$ in cultured HLE cells. These data, in conjunction with previous studies showing cataract and epithelia-specific expression of MT IIA, suggest a role for this MT isoform in protecting the lens against certain metal toxicities.

INTRODUCTION

MTs are 6-7kD metal binding proteins that function in heavy metal regulation and detoxification.[12] They are known to bind and detoxify many metal ions including: Zn, Cu, Co, Cd, Hg, Pb, Ag, Bi, Sn, Ni and Au.[12] They are rapidly induced by multiple agents in numerous tissues including the kidney,[53] liver,[53] and pulmonary epithelium.[34] In addition to their metal binding properties, they are also free radical scavengers[17] that are induced by steroids,[18,19] carcinogens,[20] chemicals that induce oxidative stress,[21] X-irradiation and UV-induced DNA damage.[22] They have been reported to function as regulators of copper homeostasis,[28-30] as zinc donors for the zinc-dependent transcription factors,[31,32] as mediators of redox balance[33] and as protectors against reactive oxygen intermediates.[34-36]

The MT family is composed of multiple, separately-encoded isoforms[37] that are grouped into four physically distinct classes labeled I- IV.[13] MTs I and II are the most widely expressed and are regulated coordinately in all tissues.[38,39] MT III is brain specific[14] and MT IV is expressed mainly in squamous epithelium.[40] In humans, single genes located on chromosome 16 and designated IIA, III and IV encode MT isoforms II-IV, while there are 13 separately encoded MT I isoforms.[40]

Metal exposure is a major form of toxicity that affects numerous non-lens tissues. In the lens, exposure to numerous metals including are Fe, Hg, Au, Ag and Cu have been associated with cataract.[1] Cataracts associated with these metals are characterized by granule formation, protein aggregation and cell death.[1] Copper exposure or altered metabolism (Wilson's disease) results in a specific form of cataract called Sunflower cataract.[1]

We have previously detected cataract[11] and epithelium-specific[54] expression of human MT IIA. In the present report we have used Northern analysis, RT-PCR and quantitative mimic RT-PCR to demonstrate that the MT IIA gene is induced in HLEs treated with $CdCl_2$ and $ZnCl_2$, but not $CuCl_2$.

METHODS

Cell culture. Human lens epithelial (HLE) cells (SRA01/04) were grown and cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, gentimycin (50 units/ml) and PSN antibody mix (50units/ml), at 37 °C in the presence of 10% CO_2.

Metal Treatment of HLE Cells. Cells were induced with heavy metals at approximately 80% confluency. Cell media was supplemented with indicated concentrations of $CdCl_2$, $ZnCl_2$ and $CuCl_2$ as metal chlorides dissolved in water. Concentrations were estimated on the basis of previous studies showing induction of MT without cell lethality. After indicated times, cells were washed with PBS and RNA isolated by Trizol (GIBCO-BRL, Gaithersburg, MD) extraction as specified by the manufacturer. Cell viability was monitored by trypan blue exclusion.[55]

RT-PCR. RT-PCR was preformed using indicated amounts of RNA using the one-step system according to the manufacturer (GIBCO-BRL, Gaithersburg, MD). Primers for MT IIA amplification were: F - 5'-AAGTCCCAGCGAACCCGCGT – 3', R-5'– CAG CAGCTGCACTTGTCCGACGC – 3'. Primers for GAPDH amplification were: F – 5' – TC CACCACCCTGTTGCTGTA and R - 5'- TGTTCCAGTATGATTC CACCC. Products were separated by gel electrophoresis on 1.5% agarose gels and visualized by

ethidium bromide staining. Products were tested to be specific for reverse-transcriptase and were sequenced to ensure specificity.

Design of PCR Mimics. The MT IIA mimic was synthesized to contain bp's +14bp to +76bp and +228bp to +250bp of the MTIIA cDNA sequence (Fig. 11). After synthesis, the mimic was re-suspended in water and used directly to compete with endogenous mRNA.

Quantitative Mimic RT-PCR. Indicated transcripts were reverse-transcribed and amplified in the presence or absence of the MT IIA PCR mimic using the One Step RT-PCR system as recommended by the manufacturer (Life Technologies, Inc., Gaithersburg, MD) and the same primers and conditions described for RT-PCR above. The MT IIA PCR mimic contains the same primer binding sites and sequence as MT IIA mRNA except that bp's 77-213 are deleted. This procedure was preformed virtually identically to that reported for PAX-6.[56] Increasing amounts (0.1 to 500pgs) of mimic DNA template competed with a constant amount of RNA(300ng) in the presence of 250μCi α^{32}P – CTP (250μCi/mmol) (Amersham, Piscataway, NJ). The resulting bands were excised from the gel and incorporated radioactivity was measured by scinilation counting. The resulting counts were corrected for background and the number of cytosine residues present in each PCR product. The amount of MT IIA transcript per ng of total RNA was determined by calculating the mimic (cpms) required to equally compete with 300ng of total RNA (cpms).

Northern Blot Analysis. Ten μg of HLE total RNA was subjected to electrophoresis on a 1% agarose gel containing 6.2% formaldehyde. The resulting gel was transferred to a nylon membrane (GIBCO-BRL, Gaithersburg, MD). After UV-cross-linking, the blot

was prehybridized in Pre-hybridization solution (GIBCO-BRL, Gaithersburg, MD) at 42°C overnight. The blot was washed three times in 0.5 X SSC, 0.2% SDS at room temperature for 20min and once in 0.25 X SSC, 0.2% SDS at 50°C for 20min. Probes were radiolabeled with α ^{32}P dCTP using the RadPrime Kit (GIBCO-BRL, Gaithersburg, MD) and hybridized for 72hrs at 50°C. The membrane was washed with 1 X SSC/0.1% SDS for 15min and then washed three times with 0.1 X SSC/0.1% SDS.

RESULTS

Induction of MTIIA by Cd, Zn and Cu.

The effects of $CdCl_2$, $ZnCl_2$ and $CuCl_2$ on MT IIA transcription were initially evaluated by Northern blotting using MT IIA cDNA as a probe. HLE cells (SRA 01/04) were grown to 80% confluency and exposed to 0.1μM of Cu^{+2} and Zn^{+2} and 0.025uM $CdCl_2$ for 8 hrs. Non-treated cells were also examined as a control. Metal-treated cells were demonstrated to be viable relative to control cells by trypan blue exclusion.[55]

Very low levels of a 0.2kb transcript hybridizing with the MT IIA probe were detected in the non-treated control RNA preparations (Fig. 7, lane 1). Cadmium (Fig. 7, lane 2) and Zn^{+2} (Fig. 7, lane 4) induced the expression of the 0.2kb transcript while $CuCl_2$ did not (Fig. 7, lane 3). Interestingly, less of the 0.2kb transcript was detected in the $CuCl_2$ treated cells than with control cells (compare lanes 1 and 3). The resulting 0.2kb bands is diffuse in the Northern blot based on the fact that it is small in size and that the MT IIA cDNA probe might also be hybridizing with other MT isoforms. Induction of MT IIA by increasing concentrations these metals was also examined by RT-PCR with gene-specific primers.[54]

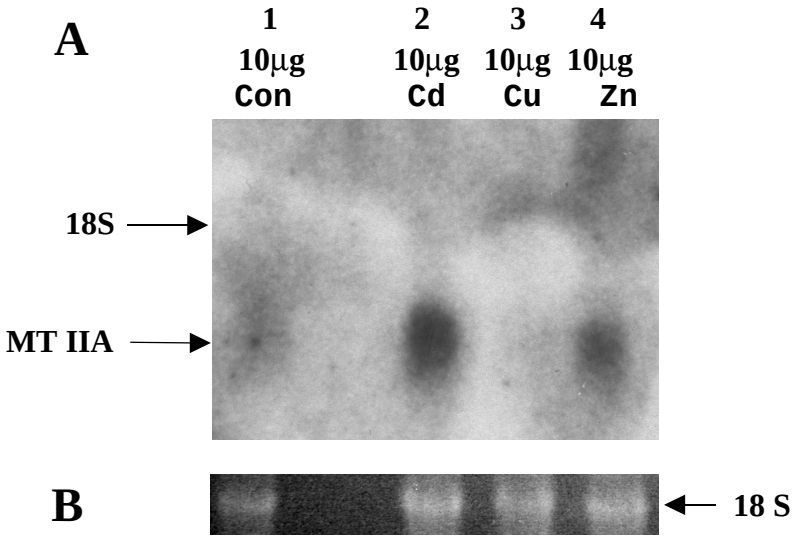

Fig. 7. A. Northern blot analysis of 10 μg of RNA isolated from cultured HLE (SRA) cells treated with 0.025μM Cd, .1μM Zn, .1μM Cu. Shown in lane 1 is the control RNA, lane 2 is CD treated cells, lane 3 is cells treated with Cu and lane 4 is cells treated with Zn. **B.** Ethidium bromide stained gel of the 18S ribosome RNA showing that equal amounts of RNA was added for each lane (1-4).

Consistent with Northern results, $CdCl_2$ and $ZnCl_2$ but not $CuCl_2$ induced MTIIA in the HLE cells relative to untreated control cells (Fig. 8). RT-PCR reactions performed with RNA from all four conditions was linear using as many as 25 PCR cycles and as much as 300ng of total RNA (Fig. 8). Based on these initial studies, 300ng and 20 PCR cycles were used in all subsequent experiments.

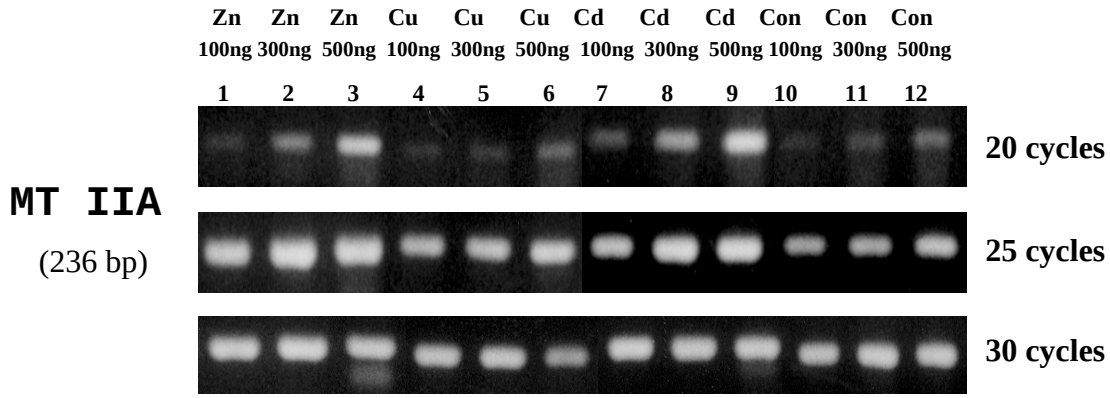

Fig. 8. Ethidium bromide stained gel showing different concentrations of HLE cell RNA's induced by either Cd, Cu or Zn. Different amounts of RNA from each metal treatment and control were subjected to RT-PCR by MT IIA specific-primers. Shown are the results of the RT-PCR for each metal treatment at different RNA levels and PCR cycles. Treatment were with 0.1 μM Zn (lanes 1-3), 0.1 μM Cu (lanes 4-6), 0.025 μM Cd (lanes 7-9) and control (lanes 10-12) are shown.

The level of MT IIA increased with increasing concentrations (0.0 μM, .025 μM, and 0.1 μM) of $CdCl_2$ and $ZnCl_2$ (Fig. 9). Maximum induction of MT IIA was observed at 0.025μM $CdCl_2$ (compare lanes 1-3) and 0.1μM (compare lanes 4-6). As control, GAPDH levels remained almost constant though a slight decrease in GAPDH was observed at 0.1μM $CdCl_2$ (lane 3). By contrast, the levels of MT IIA transcript actually decreased in the presence of $CuCl_2$ (lanes 7-9).

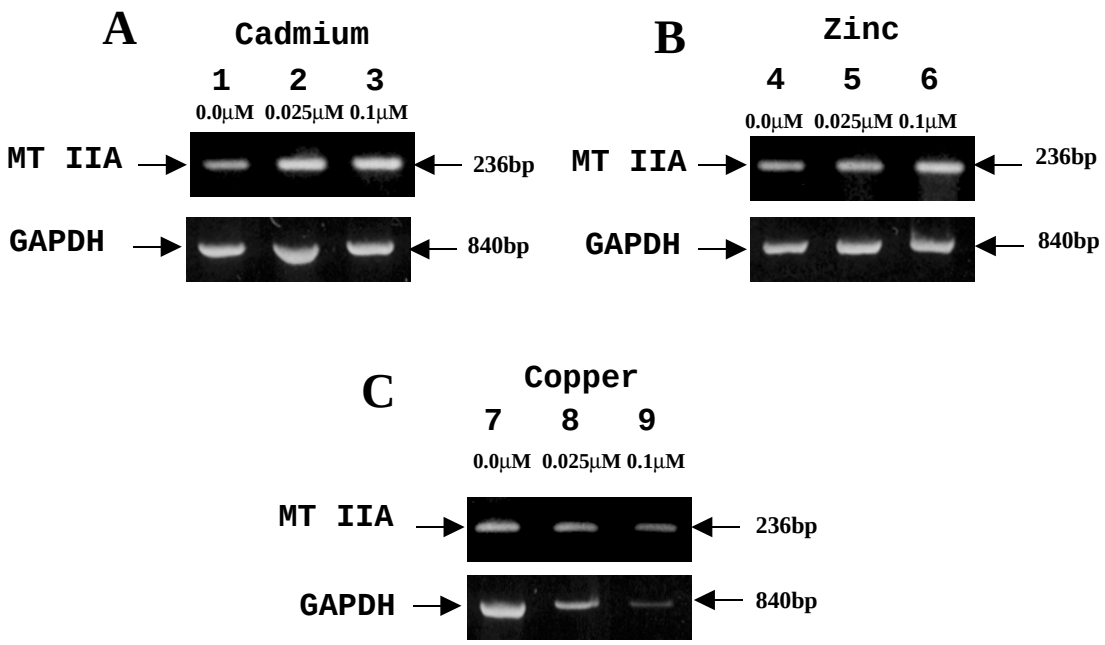

Fig. 9 Ethidium bromide stained gel showing induction of MT IIA by varying concentrations of Cd, Cu and Zn. **A.** 300 ng of RNA from each different concentration of Cd induction was subjected to RT-PCR for 20 cycles using MT IIA specific primers. Shown in lanes 1-3 are the results obtained from the different concentrations of Cd. **B.** 300 ng of RNA from each different concentration of Cu induction was subjected to RT-PCR for 20 cycles using MT IIA specific primers. Shown in lanes 4-6 are the results obtained from the different concentrations of Zn. **C.** 300 ng of RNA from each different concentration of Cu induction was subjected to RT-PCR for 20 cycles using MT IIA specific primers. Shown in lanes 7-9 are the results obtained from the different concentrations of Cu.

The optimal induction times were different for each metal (Fig. 10). From 0-8hrs after $CdCl_2$ treatment, MT IIA expression increased almost linearly (Lanes 1-7) while MT IIA was highest after 3hrs of $ZnCl_2$ treatment (lanes 8-14). The levels of MT IIA remained constant during 0-8hrs of $CuCl_2$ treatment (lanes 15-21). However, a slight decrease in MT IIA levels was detected between 3 and 8hrs of $CuCl_2$ treatment (lanes 19-21).

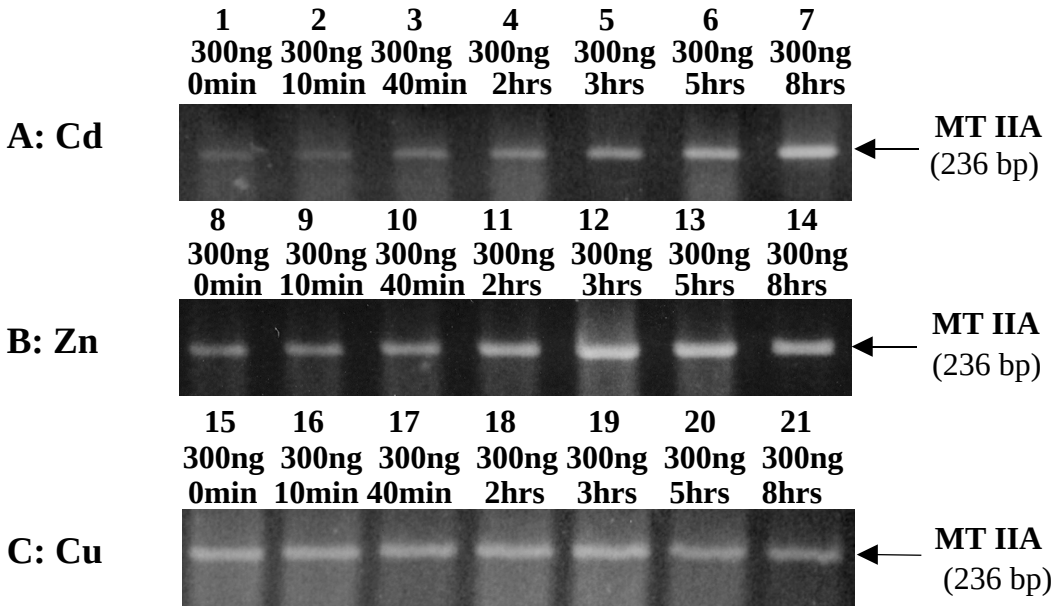

Fig. 10. Ethidium bromide stained gel showing time induction of MT IIA in HLE cells by Cd, Cu and Zn. **A**. 300ng of RNA isolated from different time induction periods were subjected to RT-PCR for 20 cycles. Shown in lanes 1-7 are the time induction results for Cd induction of MT IIA in the HLE cells from 0min – 8hrs. **B**. 300ng of RNA isolated from different time induction periods was subjected to RT-PCR for 20 cycles. Shown in lanes 8-14 are the time induction results for Zn induction of MT IIA in the HLE cells from 0min – 8hrs. **C**. 300ng of RNA isolated from different time induction periods was subjected to RT-PCR for 20 cycles. Shown in lanes 15-21 are the time induction results for Cu induction of MT IIA in the HLE cells from 0min – 8hrs.

Quantitation of MT IIA Induction by Heavy Metals

The levels of MT IIA induced by $CdCl_2$, $ZnCl_2$ and $CuCl_2$ were quantified by competitive RT-PCR. This procedure was performed using the same primers and conditions as the above procedures except that increasing amounts of a competing DNA (mimic) were added to the reaction mixture in the presence of $\alpha^{32}P$ – CTP. The competing mimic was designed to have the identical sequence as the MT IIA target cDNA except that 134 bps of internal sequence are deleted. The mimic is shown schematically in Fig. 11. The amount of mimic DNA (pg) required to equally compete

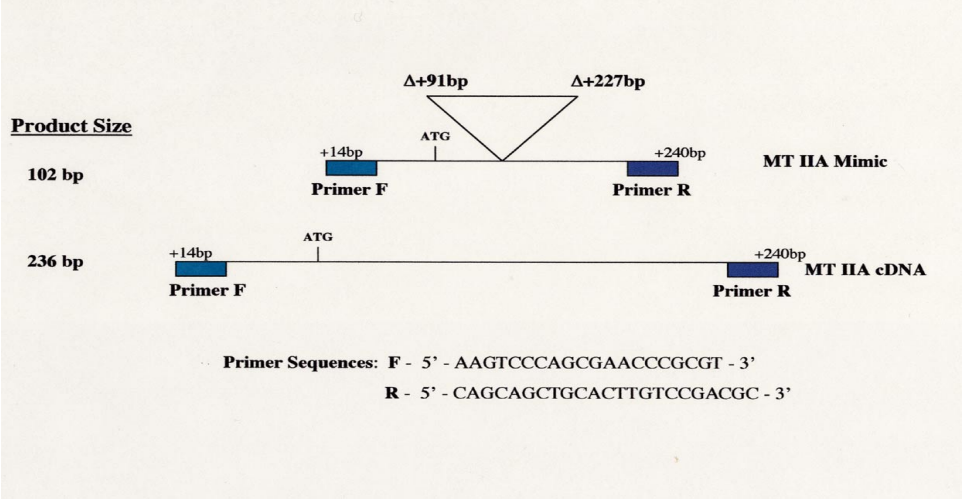

Fig. 11. Schematic of how the MT IIA mimic DNA was designed. A 136 bp internal sequence (Δ+91 to Δ+227) was deleted from the MT IIA cDNA. The bp listed are relative to the start of transcription (+1).

with 300ng of MT IIA transcript as determined by ethidium bromide staining and radioactivity is proportional to the pg of MT IIA transcript present in the 300 ng RNA sample. One to 2pgs of MT IIA mimic equally competed with the MT IIA transcript present in 300ng of RNA obtained from non-treated control cells (Fig. 12). The level of MT IIA in non-treated control cells is therefore between 0.003-0.006pgs MT IIA transcript per ng of total RNA. By contrast, the amount of mimic DNA required to equally compete with the MT IIA transcript present in 300ng of RNA from $CdCl_2$-treated cells was 10 pgs. The level of MT IIA transcript in $CdCl_2$-treated cells is therefore 0.03 pgs MT IIA transcript/ng of total RNA. The amount of MT IIA mimic required to equally compete with MTIIA transcript in 300ng of RNA from $ZnCl_2$-treated cells was between 2 to 5 pgs. Therefore, the level of MT IIA transcript in $ZnCl_2$-treated cells is between 0.006 to 0.016 pgs MT IIA transcript/ng of total RNA. Consistent with the previous Northern (Fig. 7) and RT-PCR results (Figs. 8, 9 and 10), little or no difference in the

levels of MT IIA transcript were detected between $CuCl_2$-treated cells (0.003-0.006pgs MT IIA per ng total RNA) and untreated control cells. In summary, the levels of MT IIA induction relative to non-treated control cells are: $CdCl_2$ 10 fold, $ZnCl_2$ 2-4 fold and $CuCl_2$ none.

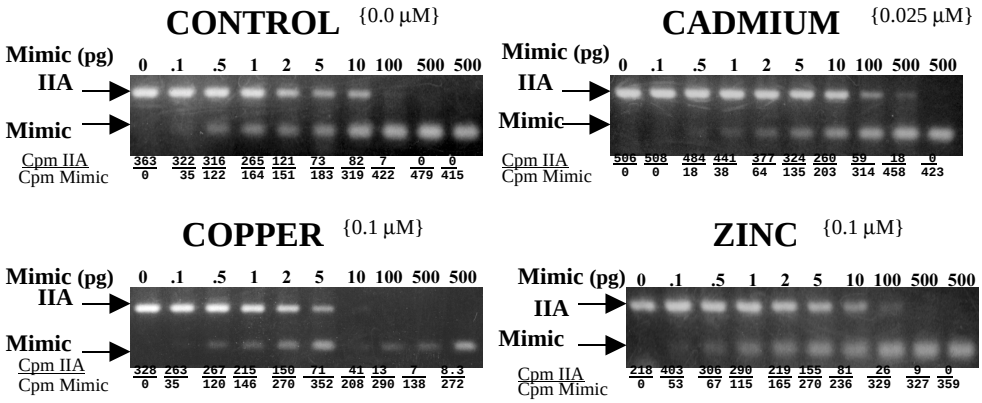

Fig. 12. Quantitative mimic RT-PCR analysis of MT IIA induction by 0.025 µM Cd, 0.1 µM Cu and 0.1 µM Zn. **A**. Ethidium bromide stained gel of 300ng control RNA amplified in the presence of 0-500pg mimic DNA and $\alpha^{32}P$ – dCTP. Indicated are the MT IIA and mimic products and the corrected radioactivity incorporated into each product. **B**. Ethidium bromide stained gel of 300ng Cd RNA amplified in the presence of 0-500pg mimic DNA and $\alpha^{32}P$ – dCTP. Indicated are the MT IIA and mimic products and the corrected radioactivity incorporated into each product. **C**. Ethidium bromide stained gel of 300ng Cu RNA amplified in the presence of 0-500pg mimic DNA and αP^{32} – dCTP. Indicated are the MT IIA and mimic products and the corrected radioactivity incorporated into each product. **D.** Ethidium bromide stained gel of 300ng Zn RNA amplified in the presence of 0-500pg mimic DNA and $\alpha^{32}P$ – dCTP. Indicated are the MT IIA and mimic products and the corrected radioactivity incorporated into each product.

DISCUSSION

My work demonstrates that high levels of both MT mRNA and protein are present in the adult human lens and establishes the identities of the individual isoforms of MT expressed by the lens. Transcripts encoding MTs IE, IF, IG, IH, IL and IIA were confirmed to be expressed by the lens (Fig. 2). By contrast, transcripts encoding isoforms IA, IB, III and IV were not detected (Fig. 2). The lack of isoform III or IV expression is not surprising, since isoform III has been previously reported to be specific for brain[14] and isoform IV has only been detected in squamous epithelium.[40] The identification of isoform IH (also known as isoform MT 0) in the adult lens is surprising since this isoform has been reported to be confined to fetal tissues.[52]

Consistent with the results obtained with the isoform-specific primers, analysis of MT expression using primers recognizing all class I and II MTs (universal primers) identified the same transcripts obtained with the isoform-specific primers (Table 1). Based on the number of specific transcripts to total transcripts detected using the universal primers, the predominance of MTs in the lens is IL> IG> IIA> IF> IH >IE. It is surprising that IL is the predominate isoform detected using the universal primers, since relatively low levels of IL were detected in using IL-specific-primers. The reason for this inconsistency is not known, but it is likely to result from differences in priming efficiency between the isoform-specific and the universal recognition primers.

The identified transcripts exhibited different spatial expression patterns between lens epithelium and lens fibers. MT I isoforms IE, IF, IG and IH (Fig. 3, lanes 1-8) were detected in both lens sub-components. Isoforms IE and IF (Fig. 3, lanes 1-4) were expressed at slightly higher levels in the lens epithelium than in the lens fibers. By

contrast, MT IIA was only detected in lens epithelium (Fig. 3, lanes 9 and 10), although low amounts of MT IIA were detected in fibers from individual lenses (Fig. 4) and with very high amounts of RNA. Due to low amplification specificity (Fig. 2), spatial analysis was not performed on isoform IL. The differences in spatial expression patterns between class I and II MTs may indicate distinct roles for these isoforms.

Consistent with the spatial expression patterns of MT transcripts detected by RT-PCR, both immunoblotting (Fig. 5) and immunostaining (Fig. 6) revealed that MT protein is mostly confined to the lens epithelium. The predominance of MT protein in the lens epithelium relative to the fibers implicates the epithelium as a primary site for possible MT function in the lens. It is interesting to note that expression of MT appears to be restricted to the outermost layers of the fibers (Fig. 6) indicating that MT expression may be restricted to those fibers that are not fully differentiated.

Significant differences in the levels and proportions of two MTs (MT IG and MT IIA) were detected in epithelia and fibers isolated from individual lenses (Fig. 4). Although further studies with large numbers of lenses will be required to establish relationships between characteristics of individual lenses and MT expression levels, the differences in MT levels detected between individual lenses in the present study appear to be independent of sex or age. Further studies will be required to establish whether these differences result from different environmental exposures, physiological conditions and/or genetic backgrounds and if these differences may be related to possible functional differences of the individual MT isoforms.

Knowing that there are six isoforms of MT expressed by the lens and that MTs exhibit differential spatial expression patterns, it is important to determine the factors that

induce MT expression in the lens. Since we have previously found that MT IIA expression is induced in cataractous lenses,[11] I wanted to determine those toxic metals that induced MT IIA expression in cultured human lens epithelial cells. I choose $CdCl_2$, $CuCl_2$ and $ZnCl_2$, since these metals are the best characterized in other cells.[42,52] The present data demonstrates that 2 toxic heavy metals can induce MT expression in cultured HLE cells (Fig. 7). Northern blotting of the RNAs obtained from the 8hrs of exposure to $CuCl_2$, $CdCl_2$, and $ZnCl_2$ shows that $CdCl_2$ was the greatest inducer of MT IIA followed by $ZnCl_2$, and that $CuCl_2$ did not induce MT IIA. Actually, there was no band in the $CuCl_2$ lane (lane 3) as compared to the control which corroborates the data presented in Fig. 9, where the message of MT IIA dropped in the different $CuCl_2$ concentrations. We also examined the steroid dexamethasone, since steroids have been shown to induce MT expression[18,19] and detected no induction of MT (data not shown).

From our initial experiments we knew that MT was induced by $CdCl_2$ and $ZnCl_2$, but these results were determined from an 8hrs exposure, so we wanted to establish the time frame that is necessary for MT induction. MT IIA induction occurs about 40min after $CdCl_2$ and $ZnCl_2$ introduction into the media but it takes at least 2hrs for $CuCl_2$ to cause a slight induction MT IIA expression. It is important to note that $CdCl_2$ was still inducing MT IIA after 8hrs and that $ZnCl_2$ induction fell off after 5hrs, while $CuCl_2$ showed no induction.

Besides determining the amount of time needed for MT induction, the minimal and maximal physiological relevant concentrations needed to induce expression was determined (Fig. 9). As little as 0.025μM $CdCl_2$ can cause induction of MT IIA expression, while concentration dependence maximizes at 0.3μM (data not shown). Cell

death began at 1.0μM (data not shown) and at even at 2.0μM $CdCl_2$, we observed cell death by trypan blue staining (data not shown). Zinc showed induction around 0.1μM concentrations and also maximized at 0.3μM (data not shown). For $CuCl_2$ inductions, MT IIA expression actually dropped by the 0.025μM and fell even more by 0.1μM. This drop in MT IIA message could be attributed to cell death because at 2.0μM concentrations we estimated, by trypan blue staining, that 43% of cells treated with $CuCl_2$ had died.

Consistent with RT-PCR experiments, quantitative RT-PCR (Fig 12) demonstrated that Cu^{+2} did not induce MT IIA expression while $CdCl_2$ increased MT IIA expression10-fold. Zinc resulted in a 3-fold induction of IIA expression (Fig. 12). Copper did not cause induction of MT IIA (Fig. 12).

Copper is known to cause cataract in the lens[1] and it is interesting to speculate that lack of MT IIA induction could be a factor. Copper deposits are involved in formation of a cataract known as the sunflower cataract.[1] Copper deposits can occur due to the use of copper-sulfate containing eye drops, copper ingestion or as a part of Wilson's disease.[1] Other metals that are involved in cataract are Cd, Fe, Hg, Au, Ag and some other metals. Most of these metals cause cataracts through granule formation and eventual cell death or protein aggregation[1]. Copper ions that are bound to amino acids or albumin and not bound to ceruloplasmin can still interact with O_2, and H_2O_2 to form OH radicals and other reactive species such as Cu(III).[57] Oxidative stress can be caused by many different insults besides metals including UV light exposure, X irradiation or natural metabolism. A defense used by the lens to combat oxidative stress is glutathione. Glutathione is present in high concetrations in the lens cytoplasm and about 12% of the ATP used by the lens is used to form the reduced (active) form of glutathione, GSH.[1] In the human lens

there is a steady decrease of GSH with age and at the same time there is an increase in cataract formation with age. Reactive oxygen species generated by the presence of $CuCl_2$ or other heavy metals could significantly affect GSH levels in the lens.

Regardless of its exact functions, the detection of increased levels of MT IIA in age-related cataracts relative to normal human lenses[11] indicates a role for MTIIA in the maintenance of lens transparency. We have also shown that MT IIA is localized to the lens epithelium while other MT isoforms are highly expressed in both lens epithelia and fibers.[54] Increased expression of MT IIA in cataract and localization of MT IIA to the lens epithelium suggests that expression of MT IIA may increase in response to exogenous insult to the lens.

My work has defined those isoforms of MT that are expressed in the lens, established their spatial expression patterns and shown that MT IIA, which is over-expressed in age related human cataract, is induced by $CdCl_2$ and $ZnCl_2$, but surprisingly not $CuCl_2$. Future studies will involve identifying those factors that regulate MT expression in the lens and the role of MTs in lens protection and cataract. My work has provided the groundwork required to carryout these studies.

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